

Competitive interaction in headwaters: slow upstream migration leads to trophic competition between native and non-native amphipods

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Abstract

The spread of non-native species is one of the outcomes of global change, threatening many native communities through predation and competition. Freshwater ecosystems are particularly affected by species turnover with non-native species. One species that has been established in Central Europe for many decades – or even a few centuries – is the amphipod crustacean *Gammarus roeselii*. Although *G. roeselii* is nowadays widespread in major river systems, there have been recent reports of its spread into smaller streams that are typically inhabited by the native species *Gammarus fossarum*. Due to their leaf shredding ability, *G. fossarum* takes up a key position in headwater streams. This raises the important question, to what extent *G. roeselii* can equivalently take over this function. To answer this question, we collected both species from nine different sites in a mid-mountain river system (Kinzig catchment, Hesse, Germany) and investigated their functional similarity using a combination of stable isotope analysis, gut content and functional morphology. The species hardly differed in morphological characteristics, only females showed differences in some traits. Gut content analysis indicated a broad dietary overlap, while stable isotopes showed a higher trophic position of *G. roeselii*. The observed functional overlap could intensify interspecific competition and allow the larger and more predaceous *G. roeselii* to replace *G. fossarum* in the future as a headwater keystone species. However, the differentiation in the stable isotopes also shows that co-existence can occur by occupying different trophic niches. Moreover, the wide range of inhabited sites and exploited resources demonstrate the omnivorous lifestyle of *G. roeselii*, which is likely to help the species succeed in rapidly changing environments.

Keywords

Freshwater ecosystems, functional morphology, *Gammarus*, gut content analysis, stable isotope analysis, trophic niche

Introduction

The introduction and spread of non-native species pose a threat to native communities globally (Gallardo et al. 2016). Due to their interconnection, streams are particularly affected by invasive species (Bij de Vaate et al. 2002; Leuven et al. 2009), which can cause a decrease of native species through predation and competition (Van der Velde et al. 2000). This change is not only expressed on a taxonomic level (Van der Velde et al. 2009), but probably also leads to changes in functional characteristics of ecosystems (Rosenfeld 2002). Non-native species might possess traits and behaviour that enable them to fulfil similar ecological functions as the native species they displace. However, the non-native species did not evolve within the recipient ecosystem and might lack natural predators, competitors or parasites that would otherwise regulate their populations in their native range (Torchin et al. 2003; Sih et al. 2010). Consequently, this unregulated population growth can lead to changes in resource availability and disrupting ecological interactions. However, post-invasion transformations of community structure and ecosystem function can differ immensely, are context-dependent and, often, poorly understood (Parker et al. 1999; Strayer et al. 2006; Kenis et al. 2009; Jeschke et al. 2014; Kumschick et al. 2015; Bellard et al. 2016).

A key group that is currently enormously affected by species-turnover is the taxonomic group of amphipods (Jażdżewski 1980; Leuven et al. 2009). Native *Gammarus* spp. are key species in freshwater ecosystems, due to their role in the decomposition of organic matter – an essential process in the headwaters of stream ecosystems (Cummins and Klug 1979; Vannote et al. 1980; Gessner et al. 1999; Graça 2001). However, the species turnover that is currently attracting attention is taking place in larger rivers (Leuven et al. 2009; Jourdan et al. 2016), while small headwaters – which are often not monitored within the Water Framework Directive – receive far less attention. While *Gammarus fossarum* Koch, 1836, is a typical headwater species, *Gammarus roeselii* Gervais, 1835, mainly inhabits the downstream parts of the rivers (Pöckl and Humpesch 1990; Pöckl et al. 2003). The exact origin and classification (native or non-native) of *G. roeselii* is still under debate, but the reduced genetic diversity (Csapó et al. 2020) and the main distribution on the Balkan Peninsula (Grabowski et al. 2017; Kabus et al. 2023) suggest that *G. roeselii* is probably an older invader in northern and western river systems outside the Danube system (Jażdżewski and Roux 1988). The species has been described near Paris in 1835 (Jażdżewski and Roux 1988), probably from a non-native population and has been spreading in Western Europe in the past centuries (Jażdżewski 1980; Jażdżewski and Roux 1988; Csapó et al. 2020). Nowadays, we observe an increasing spread of *G. roeselii* into smaller tributaries and upstream regions (Jourdan et al. 2019). In the Kinzig catchment (Hesse, Germany), some first order streams are

already colonised by *G. roeselii*, while others are still inhabited by *G. fossarum*. Interestingly, there are also streams where both occur syntopically. The similar phenotypic adaptation along environmental gradients in both species (Jourdan et al. 2019; Grethlein et al. 2022) now raises the question to which extent they share a similar trophic niche.

Investigating the trophic ecology of invasive species is necessary to gain a better understanding of the community-wide effects of invasions (Tillberg et al. 2007). Studies have shown that invaders across the animal kingdom often have a flexible and generalist diet (Garton et al. 2005; Tillberg et al. 2007; Caut et al. 2008; Zhang et al. 2010; Grey and Jackson 2012; Jackson et al. 2017; Mothapo and Wossler 2017). Especially in changing environments, omnivorous species or species with a broad trophic niche are more successful than those with a more specialised diet, so that omnivorous invasive species may prey on or compete with native species (Barbosa and Castellanos 2005; Simberloff 2010; Jackson et al. 2017).

The ecological niche of an organism is connected with its functional morphology (Bock and von Wahlert 1965). Therefore, morphological traits can provide additional important ecological insights (Premate et al. 2021). Characterising such traits – like body size and mouthparts – is suitable to evaluate the relationship between morphology and trophic ecology, which, in turn, can be tested against further results of stable isotope and gut content analyses (Premate et al. 2021). Morphological analysis combined with stable isotope analysis can be used to elucidate potential relationships between morphology and function (Hutchins et al. 2014), since trophic levels characterise the functional role of organisms (Hairston and Hairston 1993). So far, the relationship between morphology and ecology is not yet fully understood in many invertebrate groups and only now starting to become clearer in amphipods (Copilaş-Ciocianu et al. 2021).

Stable isotope analyses are useful for answering general questions about trophic structure (Vander Zanden et al. 1999; Post et al. 2000). However, this analysis is not able to distinguish very well between closely-related food sources with a similar isotopic value (McCutchan et al. 2003; Hood-Nowotny and Knols 2007; Bowes and Thorp 2015). Therefore, to gain qualitative information necessary to interpret the isotopic results, stable isotopes should be used together with other information, such as the analysis of the gut content (Inger and Bearhop 2008). This analysis has, in previous studies, been demonstrated to have a strong correlation with stable isotope data and, thus, can be considered a reliable reflection of diet in amphipods (Bacela-Spychalska and Van Der Velde 2013; Aumack et al. 2017). Copilaş-Ciocianu et al. (2021) identified a strong relationship between morphological traits and the proportion of food items found in the gut. These correlations can provide an important understanding of the functional morphology of freshwater amphipods (Copilaş-Ciocianu et al. 2021).

Stable isotope analysis is a common method used to reveal the trophic position of organisms in the field (Peterson et al. 1986; Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999; Vander Zanden et al. 1999; Post et al. 2000; Post 2002; Layman et al. 2007). This analysis is an important tool to study food webs and has already been successfully used to detect the impact of biological invasions on trophic

structures (Vander Zanden et al. 1999; Van Riel et al. 2006; Mancinelli and Vizzini 2015; McCue et al. 2020). For nitrogen, isotope ratios ($^{15}\text{N}/^{14}\text{N}$; $\delta^{15}\text{N}$) of a consumer are on average 3 to 5‰ higher than of dietary items and increase with successive trophic levels (DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987; Vander Zanden and Rasmussen 1999; Layman et al. 2007). On the contrary, the ratios of carbon isotopes ($^{13}\text{C}/^{12}\text{C}$; $\delta^{13}\text{C}$) show only minor changes since carbon moves through the food web with little alteration (DeNiro and Epstein 1978; Rounick and Winterbourn 1986; Peterson and Fry 1987; Layman et al. 2007).

We hypothesise that, in the focal study area, *G. fossarum* and *G. roeselii* share a similar trophic niche. This equivalence is suggested by a laboratory experiment where *G. roeselii* showed the same leaf consumption rate as *G. fossarum* (Jourdan et al. 2016). Moreover, we expect a high level of competition amongst ecologically similar species, which should lead to a niche shift in co-occurrence scenarios. Finally, *G. roeselii* is found both in large lowland rivers, but also occasionally in upper stretches, while *G. fossarum* is restricted to upper stretches (Pöckl and Humpesch 1990; Pöckl et al. 2003). Given that headwaters mainly receive energy input through terrestrial organic material, resulting in simpler food webs, while lower stretches exhibit more complexity due to increased nutrient availability and primary production (Vannote et al. 1980), we hypothesise that *G. roeselii* has a broader trophic niche compared to *G. fossarum*.

Materials and methods

Fieldwork

Gammarus fossarum and *G. roeselii* were collected with the kick-sampling method on 10 and 11 August 2021 at nine sampling sites in the Kinzig catchment in Hesse, Germany (Table 1; Fig. 1). The sampling sites and their site IDs correspond with the sites from Weigand et al. (2020). Adult and juvenile individuals, as well as submerged leaves of *Alnus* sp. or *Corylus* sp. (depending on availability), were collected at each sampling site. Gammarids displaying visible parasitism, such as acanthocephalans (Médoc et al. 2011;

Table 1. The nine sampling sites within the Kinzig catchment. The site ID (according to Weigand et al. (2020), name of the stream, the collected species and the GPS coordinates are given for each sampling site.

Site ID	Stream	Species	GPS
1	Gründau	<i>G. fossarum</i> + <i>G. roeselii</i>	50°14.93'N, 9°9.33'E
5	Bracht	<i>G. fossarum</i> + <i>G. roeselii</i>	50°22.62'N, 9°16.22'E
6	Bracht	<i>G. fossarum</i> + <i>G. roeselii</i>	50°26.22'N, 9°16.43'E
7	Salz	<i>G. roeselii</i>	50°25.00'N, 9°21.80'E
10	Ulmbach	<i>G. fossarum</i> + <i>G. roeselii</i>	50°20.18'N, 9°25.70'E
20	Schwarzbach	<i>G. fossarum</i> + <i>G. roeselii</i>	50°21.53'N, 9°33.11'E
33	Kinzig headwater	<i>G. fossarum</i> + <i>G. roeselii</i>	50°18.87'N, 9°36.98'E
37	Haselsbach	<i>G. fossarum</i>	50°13.87'N, 9°22.21'E
105	Riedbach, Kinzig	<i>G. fossarum</i>	50°22.62'N, 9°31.58'E

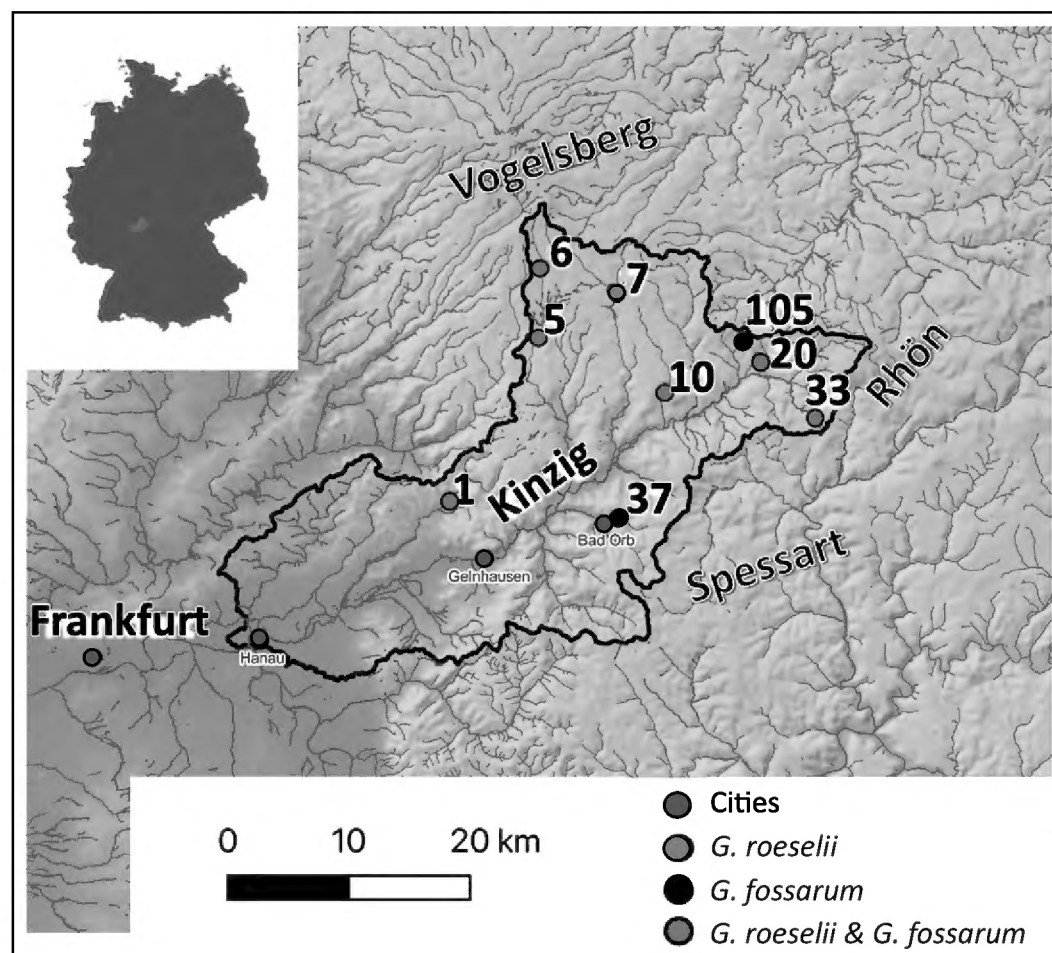


Figure 1. The nine sampling sites within the Kinzig catchment.

Kochmann et al. 2023), were omitted from the analysis due to their potential to impact the feeding behaviour and metabolic processes of the organisms.

Morphological and gut content analysis

The methodology largely followed Copilaş-Ciocianu et al. (2021). The animals were first soaked overnight in a 2% lactic acid solution and transferred to a 1:1 solution of 70% ethanol and glycerine. Dissections we performed in glycerine with the help of fine needles and microsurgical scissors. Appendages were glycerine-mounted on microscope slides. Photographs were taken using a Nikon DS-Fi2 camera attached to a Nikon Eclipse Ci-L microscope or a Nikon SMZ1000 stereomicroscope. Measurements were taken with Digimizer software (<https://www.digimizer.com/>), based on the photographs. A total of 15 female *G. fossarum*, 12 male *G. fossarum*, 14 female *G. roeselii* and 12 male *G. roeselii* were used for this analysis. We measured 35 functional morphological traits that mainly reflect the diet (body length, mouthparts, stomach and shape of gnathopods). Additionally, traits, indirectly related to diet that reflect sensory function (antennae) and locomotion (pereiopods), were measured as well. For an overview of landmarks and function of these traits, see Copilaş-Ciocianu et al. (2021). The same individuals used for the morphological analysis were used for the gut content analysis. The gut content analysis followed the protocol of Copilaş-Ciocianu et al. (2021), using a Nikon Eclipse Ci-L microscope. In short, the gut was emptied out and evenly spread on a glass slide with a square grid (24 × 24 mm) containing 10 × 10

smaller squares. The gut content was classified into six categories (alga, arthropod, detritus, fungus, plant, sand) and the number of squares on which a particular food item occurred were counted to calculate proportions.

Stable isotope analysis

Two gammarids within each 2 mm size class were used for the analysis. Amphipods with a body size lower than 7 mm were considered juvenile. In total, 26 juveniles, 18 females and 24 males of *G. fossarum* and 19 juveniles, 36 females and 28 males of *G. roeselii* were used. Three leaf replicates per site were used as baseline for trophic position estimates. All samples were sorted, washed with distilled water and dried for 48 h at 60 °C. Afterwards, they were ground to a fine powder with a pestle and mortar. The powder for each amphipod sample was aimed to be 1 mg. In case juveniles did not have a high enough body mass, a composite sample of more individuals of the same species, sex and size class was used. The aimed weight for the leaves was between 3 mg and 4 mg powder for each sample. Carbon and nitrogen stable isotope ratios were measured at the Isotopic Research Laboratory of the Centre for Physical Sciences and Technology in Vilnius, Lithuania. Here, an elemental analyser coupled to the isotope ratio mass spectrometer (EA-IRMS, Flash EA1112–Thermo V Advantage) via the ConFlo III interface was used for the measurement.

In our amphipod samples, the C:N mass ratio varied over 3.5 and in our leaf samples the C exceeded 40%; thus, we corrected the $\delta^{13}\text{C}$ values for lipid content using the relevant formulae for aquatic animals and plants from Post et al. (2007). We referenced the trophic position of amphipods to ΔC and ΔN in each site by subtracting the corresponding mean values of the leaves. We further divided the ΔN by the conventional trophic fractionation factor value of 3.4‰ (Post 2002) and added one (baseline trophic level) to obtain the amphipod trophic level (TL) estimates.

Statistical analysis

To correct for body size, the morphological measurements were first regressed against the body length and the residuals across all sampling sites were pooled into four species by sex groups (female *G. roeselii*, male *G. roeselii*, female *G. fossarum*, and male *G. fossarum*). The mean value of residuals was used in case of missing values. The gut content data of all sampling sites were grouped in the same manner. Subsequently, principal component analyses (PCA) were conducted in PAST 4 (version 4.08; Hammer et al. 2001). They were computed with a correlation matrix. To test for dietary or morphological differences amongst groups, a one-way permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations and Euclidean similarity index was conducted in PAST. The PERMANOVA was performed either on the gut content data or all morphological traits combined or separately on four trait-complexes reflecting sensorial function (antennae, six measurements), food grasping and manipulation (gnathopods, eight measurements), food processing and digestion (mouthparts and

stomach, four measurements) and locomotion (pereiopods along with their accompanying bases and coxae, 15 measurements). To test for differences between gammarids occurring alone compared to co-occurrence, PERMANOVA was performed on gut content and all morphological traits between those sites. Bonferroni correction was applied for further multiple comparisons between group pairs.

We analysed the difference in trophic position between species by building linear mixed-effects models (LMEMs) for each of the referenced metrics (ΔC and TL). In these models, we considered the interacting fixed effects of size, species and the syntopy (or co-occurrence) factor, while site was included as a random factor. The continuous size variable was centred around the global mean of 8.25 mm for more proper effect testing (but back-transformations were applied for the provided visuals). The effects were tested using type III analysis of variance with Satterthwaite's approximation for denominator degrees of freedom. These analyses were conducted by employing the R packages *lme4* v. 1.1-32 and *lmerTest* v. 3.1-3 (Kuznetsova et al. 2017) and visualised using the package *visreg* v. 2.7.0 (Breheny and Burchett 2017). We further conducted the *post hoc* group comparisons at minimum and maximum amphipod sizes with the Šidak *p*-value adjustment for eight tests using the package *emmeans* v. 1.8.8 (Lenth 2023). To test for the differences in ΔC and TL between sexes, we extracted the residuals from the LMEMs for the adult observations only and applied simple linear models with backward stepwise removal of terms (based on Akaike Information Criterion), starting with fully interacting effects of species, sex and syntopy.

In the bivariate stable isotope space, we approximated the population isotopic niches as ellipses containing 95% of the data with their area estimates ($BEA_{95\%}$). We also estimated the overlaps between the species in the six syntopic sites and standardised them as proportions of the sum of the non-overlapping ellipse areas (0 – no overlap, 1 – complete overlap). For this, we used the Bayesian estimation available in R package *SIBER* v. 1.2.7 (Jackson et al. 2011) and provided the estimates as modes with 95% credible intervals of the posterior distributions (400 draws). Using these draws, within each of the six syntopic sites, we tested if: (1) the $BEA_{95\%}$ of *G. roeselii* is wider than the ellipse of *G. fossarum*, (2) if the overlap is larger than 0 and (3) if the overlap is larger than 60% which is the commonly considered threshold for an ecologically significant overlap (Jackson et al. 2011). Provided probabilities were translated to be treated conventionally – significant effects when $p < 0.05$.

Results

Morphological analysis

Results from omnibus PERMANOVA testing for all traits ($F = 12.7$, $p = 0.001$) revealed a significant morphological differentiation between sexes within *G. roeselii* and within *G. fossarum* and between females of both species, but not males (see Suppl. material

1: table S1 for pairwise comparisons). This could be confirmed by the PCA, since the convex hulls of the males of both species are mainly overlapping, whereas the females are more separated from each other (Fig. 2a). Pairwise comparisons (Suppl. material 1: table S1, Fig. 2b–e) further showed that females differed significantly between species only with regard to gnathopods and pereiopod+coxae trait complex (Fig. 2c, d). The pairwise comparisons also indicated that the sexual dimorphism within both species was reflected by significant differences in all tested trait complexes. Regarding the comparison between gammarids occurring alone compared to co-occurring, the omnibus PERMANOVA testing for all traits (*G. fossarum* alone/co-occurring: $F = 0.54$, $p = 0.64$; *G. roeselii* alone/co-occurring: $F = 0.96$, $p = 0.38$) revealed no significant morphological differentiation.

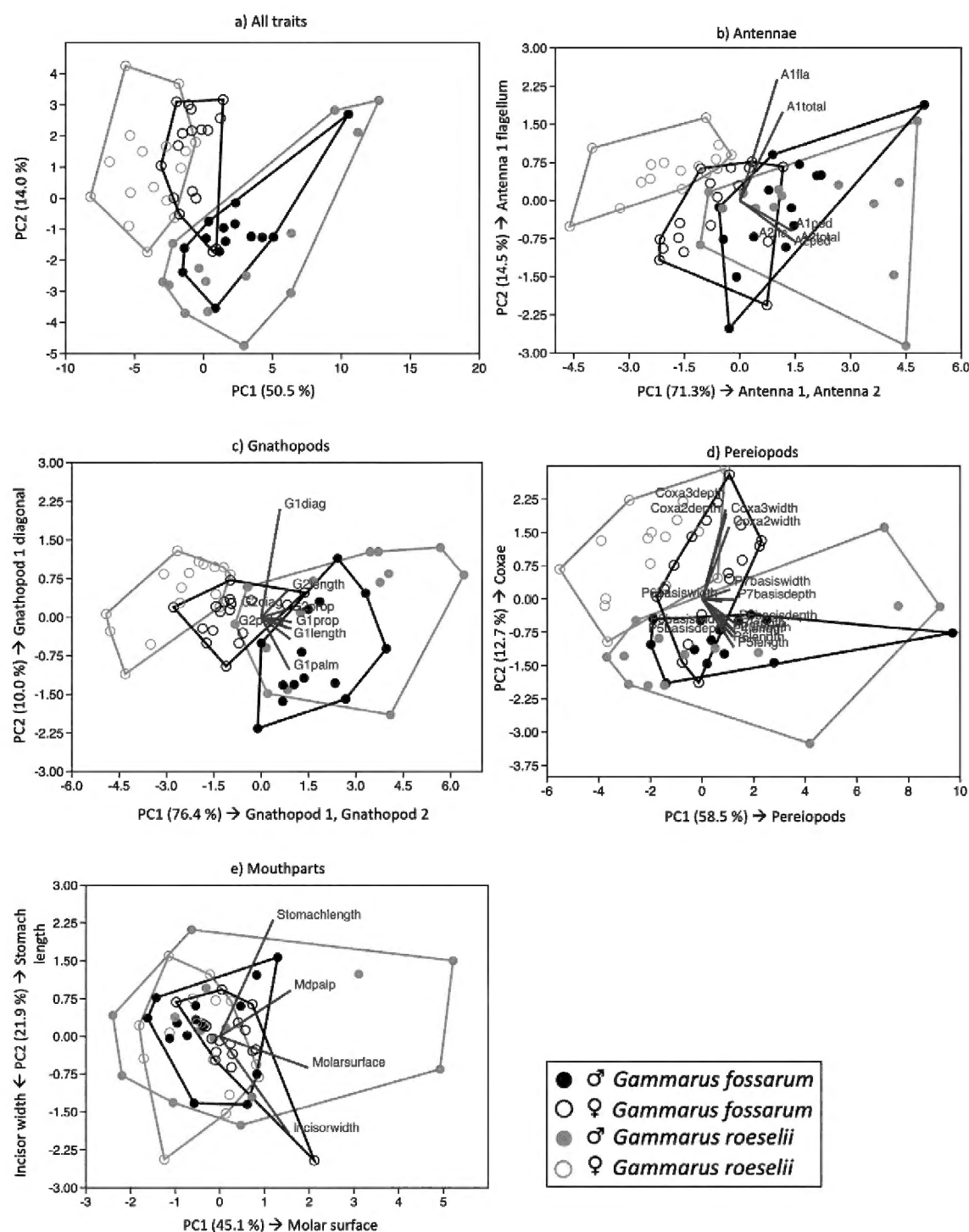


Figure 2. Morphological differentiation between studied male and female gammarid species according to principal component analyses (PCAs) of all traits (a), antennae (b), gnathopods (c), pereiopods+coxae (d) and mouthparts (e).

Gut content analysis

The PCA of the gut content indicated that the most important differentiation between specimens was amongst the detritus, sand vs. plant axis, explaining 29.9% of variation (Fig. 3). The second axis of differentiation was between detritus and sand and it explained 21.4% of the variance (Fig. 3). The PERMANOVA revealed no significant differentiation between species or sexes when only considering co-occurrences ($F = 1.3$, $p = 0.24$). All groups overlapped to a great extent (Fig. 3). Results from PERMANOVA testing for gut content between syntopic and non-syntopic populations did reveal a significant differentiation between *G. fossarum* alone/co-occurring with *G. roeselii* ($F = 3.6$, $p = 0.032$), but not between *G. roeselii* alone/co-occurring with *G. fossarum* ($F = 2.6$, $p = 0.065$, Fig. 3b).

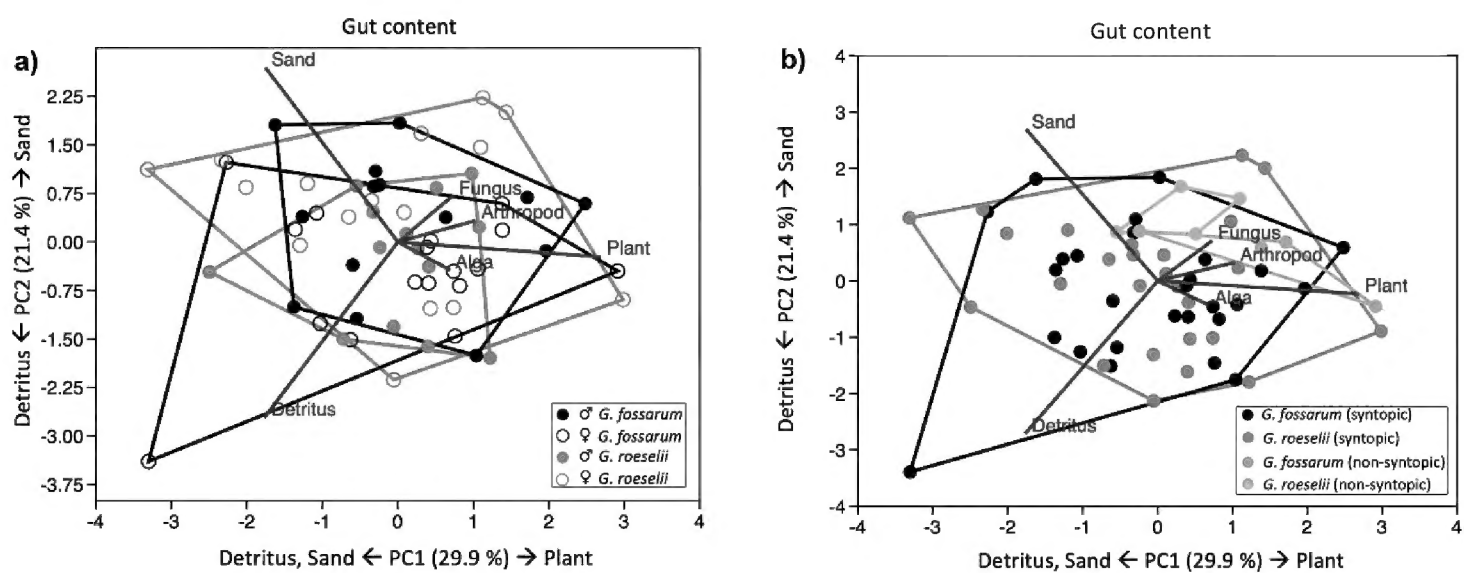


Figure 3. A principal component analysis (PCA) scatterplot depicting dietary differentiation with respect to the proportion of various food items of species by **a** sex and **b** by non-syntopic and syntopic occurrence (sexes combined).

Stable isotope analysis

Isotopic position

The results of stable isotope analysis revealed pronounced niche differentiation between *G. fossarum* and *G. roeselii*, with *G. roeselii* generally occupying a higher trophic level (Fig. 4). At most sites, ΔC values varied indicating ^{13}C -enrichment of amphipods relative to the tree-leaf detritus. However, we observed a notable separation of site 7, where *G. roeselii* was exceptionally ^{13}C -depleted relative to the leaves (coincidentally, *Alnus* sp. leaves were missing at the site, thus *Corylus* sp. were used). Trophic level of amphipods mostly fell within the normal range between herbivory (TL = 2) and complete carnivory (TL = 3), with the extremes of 1.8 in *G. fossarum* juveniles from site 37 and slightly above 3.0 in the largest adults of *G. roeselii* from site 6 and site 10. The trend of increasing TL with size was also reflected in the data. Estimates of trophic

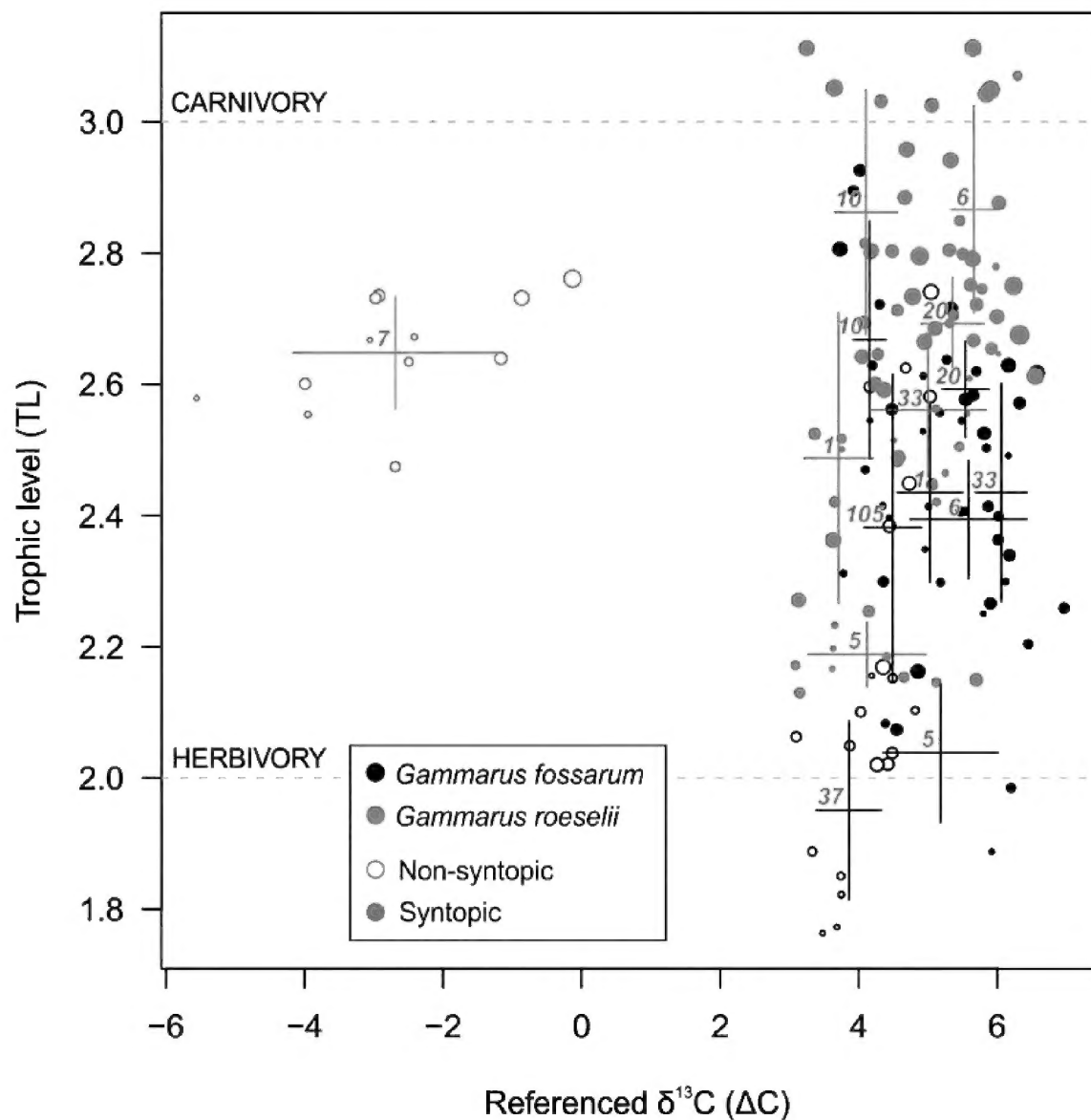


Figure 4. Referenced stable isotope biplot of studied amphipods showing trophic niches by site (means \pm SD). Point size reflects animal body size. Green labels at the means correspond to site IDs from Table 1.

position for different amphipod size groups derived from stable isotope analysis are provided in Suppl. material 1: table S2.

Both LMEMs of ΔC and TL (Table 2) returned a significant second order interaction of amphipod size, species and the syntopy factor, indicating variable steepness of the ontogenetic slopes of gammarids. Judging by the various modelled cases (Fig. 5), there was always an increasing ΔC and TL trend with amphipod size and *G. roeselii* generally tended to exhibit lower ΔC and higher TL values than *G. fossarum*. Regarding both metrics, the interspecific differences in the overall position, as well as the slopes, were more evident across the non-syntopic sites, while, when co-occurring, the two species tended to overlap more and to exhibit very similar ontogenetic slopes. Across the syntopic sites, both amphipods appeared to have high ΔC values, similar as in *G. fossarum*-only sites and their juveniles converged more to intermediate TL values. The ΔC slope of *G. roeselii* and TL slope of *G. fossarum* were visibly steeper across the non-syntopic sites in comparison to the syntopic ones, suggesting wider ontogenetic niches in respective metrics when a single species is found. (It is important to note that, to some extent, the observed patterns may have been biased by the single *G. roeselii*-only site coinciding with a more degraded habitat.)

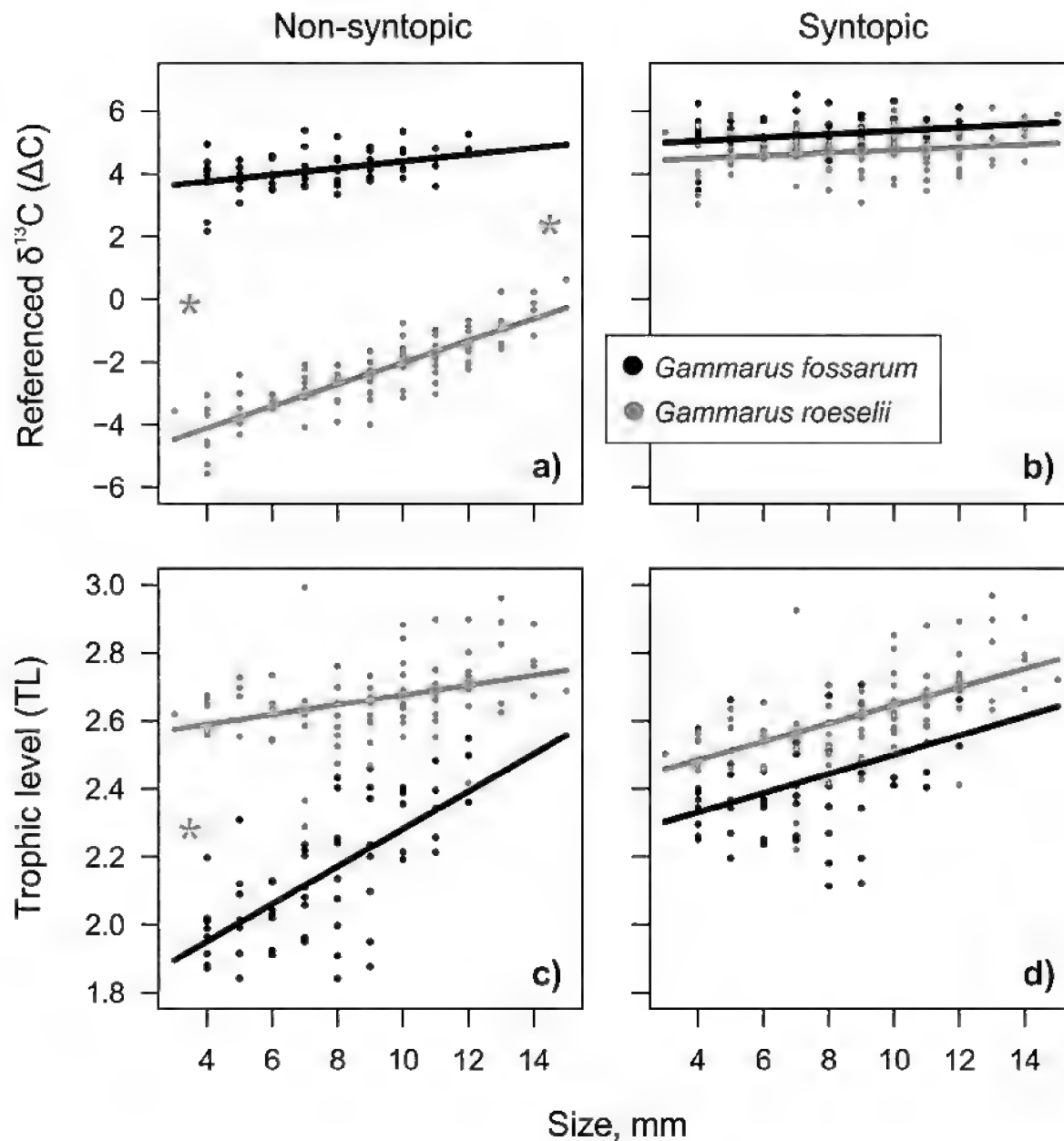


Figure 5. Effects of amphipod size, species and their syntopic occurrence on isotopic metrics of trophic position within the linear mixed-effects models of **a, b** referenced $\delta^{13}\text{C}$ (ΔC) and **c, d** of trophic level (TL) by **a, c** non-syntopic vs. **b, d** syntopic sites. See Table 2 for effect tests. Grey asterisks between species' lines indicate significant ($p < 0.05$) interspecific differences at corresponding size extremes according to *post hoc* analysis. Not shown here, but this analysis also indicated significant ΔC differences within the smallest and largest specimens of *G. roeselii* occurring in non-syntopic vs. syntopic sites.

Table 2. Results of analysis of variance (type III decomposition) from the linear mixed-effects models of isotopic metrics of trophic position – referenced $\delta^{13}\text{C}$ (ΔC) and trophic level (TL) – testing for the interacting effects of amphipod size, species (*Gammarus fossarum* vs. *G. roeselii*) and their syntopic occurrence. See Fig. 5 for effect plots.

Tested term	df	ΔC model			TL model		
		df _{denominator}	F	p	df _{denominator}	F	p
Size	1	142.6	35.2	< 0.001	142.1	43.4	< 0.001
Species	1	10.6	125.1	< 0.001	9.4	7.0	0.026
Syntopy	1	9.7	114.8	< 0.001	9.1	0.6	0.459
Size : Species	1	142.7	6.3	0.013	142.1	5.0	0.027
Size : Syntopy	1	142.6	14.7	< 0.001	142.1	0.6	0.444
Species : Syntopy	1	10.6	87.8	< 0.001	9.4	1.9	0.202
Size : Species : Syntopy	1	142.7	7.1	0.008	142.1	4.3	0.039

Regarding the effect of sex across the adult dataset, the stepwise procedure removed all the effects apart from sex from the linear model of ΔC and indicated a model without predictors for TL (although sex was removed last). Thus, we ended up applying simple t -tests using only the sex factor. These indicated a marginally higher female ΔC ($t_{104} = 1.9, p = 0.061$), but no effect of sex on TL ($t_{104} = 0.4, p = 0.69$).

Isotopic niche width and overlaps

Isotopic niche widths of the populations and the overlaps between species are provided in Table 3. Although the mode estimates of $BEA_{95\%}$ were often larger in *G. roeselii* than in *G. fossarum*, the Bayesian testing indicated such a pattern significantly only in site 10 ($p = 0.030$ as opposed to $p \geq 0.84$ in other cases). All the six overlaps were larger than 0 ($p \leq 0.037$), but none of them exceeded 60% ($p \geq 0.96$), indicating a relatively low trophic niche overlap.

Table 3. Population isotopic niche widths as ellipse areas ($BEA_{95\%}$) and their absolute and relative overlaps by study site. The Bayesian estimates are provided as modes and 95% credible intervals.

Site ID	<i>G. fossarum</i> $BEA_{95\%}$	<i>G. roeselii</i> $BEA_{95\%}$	Overlap	%Overlap
37	3.47 (1.56–6.59)	-	-	-
105	5.20 (3.01–9.21)	-	-	-
6	3.51 (2.10–6.79)	3.05 (1.96–5.37)	0.02 (0.00–1.44)	0.00 (0.00–0.20)
10	2.10 (0.99–4.07)	4.76 (3.09–9.63)	1.61 (0.46–2.88)	0.26 (0.08–0.49)
20	1.31 (0.66–2.94)	1.93 (1.34–3.67)	0.70 (0.00–1.47)	0.27 (0.00–0.55)
33	3.53 (2.09–8.38)	4.85 (3.10–8.40)	1.73 (0.15–3.17)	0.24 (0.05–0.39)
1	3.63 (1.56–9.25)	4.78 (1.79–11.19)	0.04 (0.00–3.30)	0.00 (0.00–0.32)
5	3.84 (1.94–10.98)	2.39 (1.42–4.85)	1.19 (0.00–2.50)	0.02 (0.00–0.40)
7	-	6.75 (3.66–12.41)	-	-

Discussion

Our study revealed a strong overlap in morphology and gut content between the native *G. fossarum* and non-native *G. roeselii* in headwater streams. However, stable isotopes indicated a stronger dietary differentiation between the two species when occurring alone and a more similar trophic niche when occurring together, with *G. roeselii*, however, generally occupying a higher trophic level. In addition, the gut content analysis confirmed that *G. fossarum* appears to have a different diet when occurring alone. This indicates that, despite their apparent functional morphological equivalence, the two species exploit different food resources. Below, we expand on the significance of these findings.

Overall, morphological differentiation occurs between the sexes rather than between species when looking at the combined data, pooled over all sampling sites. Only in some traits of the females (gnathopods, pereopods and coxae) could we see differences between the species. This differentiation between females of both species could be explained by different reproduction characteristics of the species, such as different thermal optima for maximum fecundity (Pöckl 1993). As reproduction involves a met-

abolic cost (Sutcliffe 1992), a different reproduction effort could mean that different energy levels are available for growth and, thus, translating into different sizes of morphological features. Besides, the size of males is significantly affected by sexual selection, while females are significantly affected by natural selection (Ward 1988). Sexual selection of a trait is induced by competition over mates (Andersson 1994), while natural selection is induced by trait variation amongst individuals (Endler 1986). The competition between males of both species might be similar throughout the sampling sites and, therefore, the males show similar morphological traits. On the other hand, females of both species experiencing natural selection might lead to morphological trait differentiation. Similar morphological features of males of both species point to an absence of morpho-functional differentiation, the occupation of a similar trophic niche and ecological similarity (Cothran et al. 2013; Fišer et al. 2015; Copilaş-Ciocianu et al. 2021). However, morphological similarity does not always mean ecological equivalence. Fišer et al. (2015) found ecological differentiation between four morphologically similar *Niphargus* species, which could potentially lead to different ecological roles in the ecosystem. Moreover, Premate et al. (2023) discovered that morphological traits are influenced not only by trophic position, but also by the specific habitat type. This observation could also explain the similar morphology of our study species occurring in the same headwater habitat.

The gut content analysis showed a strong dietary overlap between the species when pooled over all sampling sites. Our results thus indicate that the foraging on the same food sources might lead to competition between the two species. In principle, a strategy adopted by different amphipods to reduce competition for limited resources could be to utilise resources in different ways, in different microhabitats or at different times (Piscart et al. 2010, 2011; Mauchart et al. 2017; Premate et al. 2021). Apart from that, our species differed significantly regarding the $\delta^{13}\text{C}$ level, which has also been observed in other co-occurring freshwater amphipods (Premate et al. 2021). Such partitioning of food resources is expected amongst co-occurring species (Schoener 1974; Chesson 2000), facilitating a stable co-existence (Chesson 2000). Our analysis showed a significant differentiation in gut content for *G. fossarum* when occurring alone compared to co-occurring with *G. roeselii* and close to significance for *G. roeselii* when occurring alone compared to co-occurring with *G. fossarum*. This could be explained by their, in general, separated occurrence in different river sections (Pöckl and Humpesch 1990; Pöckl et al. 2003) and the different availability of resources in these sections (Vannote et al. 1980). On the other hand, the absence of dietary separation for co-occurring populations might be a consequence of high abundance of food during the study period and could indicate potential for interspecific competition (Piscart et al. 2011; Rothhaupt et al. 2014). Interspecific competition, in turn, can reduce intraspecific specialisation, therefore, reducing the niche breadth of individuals (Araújo et al. 2011), leading to a dietary overlap. In addition, dietary separations or overlaps can change frequently in populations depending on spatio-temporal availability of food items and on the abundance of competing species (Rothhaupt et al. 2014). Thus, stable isotope analysis is useful for studying the diet on a longer time-scale.

The stable isotope analysis revealed that both species had more similar trophic niches when they co-occurred. Specifically, *G. fossarum* underwent a trophic level increase while *G. roeselii* a decrease which was also accompanied by a shift in the carbon source. Although this result was unexpected and non-intuitive at a first glance, it could be explained by reciprocal predation on juveniles or recently moulted individuals of the other species, as observed in another native and non-native amphipod species pair (Dick et al. 1993; Dick 1996; Dick and Platvoet 1996). Moreover, other researchers have discovered niche segregation in co-occurring amphipods in order to facilitate co-existence and avoid competition (Piscart et al. 2011; Premate et al. 2021).

Nevertheless, our stable isotope analysis revealed that, even though both species experience a niche shift, *G. roeselii* still has generally a higher trophic position compared to *G. fossarum* when both species occur together, contradicting our first hypothesis of a shared trophic niche. One reason why we have not found this more carnivorous lifestyle in the gut content could be that animal material can be digested more quickly (Guerra-García et al. 2014), but the stable isotopes of animal prey are incorporated in the tissues of the amphipod and, thus, influence the stable isotope analysis. The higher trophic position of the non-native *G. roeselii* observed in our stable isotope analysis could be an advantage compared to the native *G. fossarum*. Animal material, a high quality and easily-assimilated food item, can improve the growth of *Gammarus* sp. (Anderson and Cummins 1979; Crenier et al. 2017). The acceleration of growth, maturation and reproduction progress when being fed animal matter has been observed for several *Gammarus* species (Vassallo and Steele 1980; Smith 1988; Delong et al. 1993). In addition, it has been observed for *G. fasciatus* that, with increasing size, the amount of animal matter found in the gut increased proportionally (Summers et al. 1997). The present study showed that larger individuals occupy a higher trophic niche, confirming an ontogenetic niche shift. Thus, since *G. roeselii* usually has a larger body length, it will presumably consume more animal material than the smaller *G. fossarum* (DeLong et al. 1993). In return, individuals of *G. fossarum* have a lower trophic position than *G. roeselii*. Since *G. fossarum* also has a smaller body size and smaller gnathopods than *G. roeselii*, it may be mainly limited to detritus because their smaller gnathopods may not be large enough to handle larger food items, such as animal matter (Summers et al. 1997). This is supported by a laboratory experiment of Delong et al. (1993), which showed that small individuals of *G. fasciatus* showed a delayed growth response when fed animal matter, but a normal growth when fed only leaf litter. However, *G. fossarum* is generally also known to be predatory and able to exploit animal food resources (Stoffels et al. 2011; Georgievová et al. 2020; Syrovátka et al. 2020) resulting in gammarids generally being classified as rather omnivorous (MacNeil et al. 1997).

Overall, our results indicate that *G. roeselii* exhibits a broader trophic niche than *G. fossarum* confirming our second hypothesis. It has been shown that, when *G. roeselii* co-occurs with *G. fossarum*, it significantly affects their micro-distribution (Mauchart et al. 2017). *G. roeselii* chooses habitats with macrophytes, (dead) wood or deposits of CPOM (coarse particulate organic matter), whereas *G. fossarum* prefers coarse gravel and cobbles (Mauchart et al. 2017). This suggest different sources of algal or terrestrial

$\delta^{13}\text{C}$ (Finlay 2001, 2004) for the diet of *G. roeselii* and could be an explanation for its broader trophic niche. Moreover, in our stable isotope analysis, population from site 7 appears to be an outlier with lower $\delta^{13}\text{C}$ values. Site 7, unlike the other sites, was highly morphologically degraded, canalised and without significant riparian vegetation. Unlike *G. fossarum*, *G. roeselii* is able to occur in many anthropogenically shaped habitats (Mauchart et al. 2014; Enns et al. 2023; Kochmann et al. 2023). Apparently, in such habitats, *G. roeselii* is able to exploit resources from different food chains, such as algae. On the other hand, the larger size of *G. roeselii* could be an explanation for its broader trophic niche, because the larger size can offer competitive advantage (Young 2004). This advantage arises from the potential for a wider dietary range as size increases, potentially resulting in accelerated growth rates and shorter reproduction times (Summers et al. 1997). Moreover, it enables the exploitation of seasonal changes of food items (DeLong et al. 1993). High trophic diversity is one of the most important factors responsible for the dispersal success of amphipods (Legeżyńska et al. 2012), because invasive species must be able to gain food material in heterogeneous habitats (Rothhaupt et al. 2014). Nevertheless, in a future study, it needs to be elucidated whether *G. roeselii* has a similar or different niche in their native range on the Balkan Peninsula. Presumably, *G. roeselii* could become even more competitive through depending on leaf litter as constant adaptation, effectively exploiting leaf litter, and also higher quality food sources and continuing to colonise tributaries and headwaters (Jourdan et al. 2016).

The omnivorous diet of both amphipods, the higher trophic niche of *G. roeselii* seen in our stable isotope analysis and the observed microhabitat partitioning in the field (Mauchart et al. 2017) can facilitate and explain the co-existence of both species. However, unlike *G. fossarum*, *G. roeselii* possesses dorsal pleosomal spines. An experimental study identified these as an effective defence mechanism against predatory fish (Bollache et al. 2006). The protection through spines can probably not only facilitate its spatial distribution, but may also favour the colonisation of new niches, hence increasing its invasion potential (Copilaş-Ciocianu et al. 2020). The low predation of *G. roeselii* may promote its establishment in communities with native species, such as *G. fossarum* (Bollache et al. 2006). Moreover, headwater sections of streams are particularly impacted by anthropogenic pollution and a subsequent species turnover (Betz-Koch et al. 2023; Enns et al. 2023). This ongoing pollution can facilitate the spread and establishment of *G. roeselii* into headwaters (Mauchart et al. 2014; Jourdan et al. 2024) and can lead to the decline of the more sensitive *G. fossarum* (Enns et al. 2023). In addition, temperature records for the herein studied sites show water temperatures between 14.4 °C and 21.5 °C, which are congruent with an earlier study of some of the sites (Grethlein et al. 2022). *G. roeselii* reaches sexual maturity faster, has a shorter brood development and a higher reproductive success than *G. fossarum* at temperatures above 12 °C (Pöckl et al. 2003). Furthermore, these characteristics of *G. roeselii* are enhancing with increasing temperature (Pöckl 1992). These life-history traits could facilitate invasion success and alter the community structure of invaded habitats (Grabowski et al. 2007; Rothhaupt et al. 2014). Thus, due to increasing water

temperatures associated with climate change and ongoing pollution through anthropogenic impact, *G. roeselii* might outcompete *G. fossarum* in headwaters in the future (Pöckl et al. 2003; Enns et al. 2023; Jourdan et al. 2024).

Conclusions

Our study revealed that the non-native *G. roeselii* is morphologically similar to the native *G. fossarum* in headwater streams. We also found similar food items in the gut content, which showed the generally omnivorous lifestyle of both species. However, stable isotopes indicated that the trophic niches of both species differ substantially, with *G. roeselii* being more predaceous and generally having a broader niche. This indicates that, despite their shared morphological characteristics and omnivorous tendencies, there is a noticeable niche differentiation in *G. roeselii*, consequently influencing the headwater food web. In situations of ample resource availability, co-existence between both species may be possible. However, in cases of resource scarcity, we expect *G. roeselii* to be competitively superior, particularly given its ability to exploit a broader range of food resources, regardless of their quality.

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Supplementary material I

Supplementary information

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Data type: docx

Explanation note: **table S1.** Results (*p*-values) of the PERMANOVA for the morphological traits of GR-F (female *G. roeselii*), GR-M (male *G. roeselii*), GF-F (female *G. fossarum*) and GF-M (male *G. fossarum*). PERMANOVA was performed with 9999 permutations and Euclidean similarity index. Bonferroni correction was applied for multiple comparisons between group pairs. Significant *p*-values (< 0.05) are marked in bold. **table S2.** Trophic position metrics of different amphipod size groups by study site (derived from stable isotope analysis relative to tree-leaf detritus). Provided values are means with standard deviations.

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